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Abstract

Background: The link between environmental estrogen exposure and defects in the female reproductive tract is well established. The phytoestrogen genistein is able to modulate uterine estrogen receptor (ER) activity and dietary exposure is associated with uterine pathologies. Regulation of stress and immune functions by the glucocorticoid receptor (GR) is also an integral part of maintaining reproductive tract function and disruption of GR signaling by genistein may also have a role in the adverse effects of genistein.

Objective: We evaluated the transcriptional response to genistein in Ishikawa cells and investigated the effects of genistein on GR-mediated target genes.

Methods: Ishikawa cells were used as a model system to identify novel targets of genistein and the synthetic glucocorticoid dexamethasone through whole genome microarray analysis. Common gene targets were defined and response patterns verified by quantitative real-time reverse transcription-PCR. The mechanism of transcriptional antagonism was determined for select genes.

Results: Genistein regulates numerous genes in Ishikawa cells independently of estradiol, and the response to co-administration of genistein and dexamethasone is unique compared to estradiol and dexamethasone. Furthermore, genistein alters glucocorticoid regulation of GR-target genes. In a select set of genes, co-regulation by dexamethasone and genistein was found to require both GR and ERα signaling, respectively.

Conclusions: Using Ishikawa cells, we observed that exposure to genistein results in distinct changes in gene expression and unique differences in the glucocorticoid receptor transcriptome.

Introduction

Environmental compounds with estrogenic activity, present in plant constituents, plastics, and pesticides, are recognized endocrine disruptors, leading to impaired reproductive function in a number of species (Caserta et al. 2008). Some of these compounds display a similar structure to the natural ligand and are able to physically interact with the estrogen receptors (ER), mimicking the activity of estradiol (Gray et al. 2002). However, unlike the biological effects of estradiol, which are regulated by feedback from the hypothalamic-pituitary-gonadal axis, dietary exposure to phytoestrogens is not under the control of feedback mechanisms and can potentially negatively impact reproductive tract function (Christensen et al. 2012). Due to the reported health benefits, consumption of soy in the United States has increased since the early 1990's (Adlercreutz et al. 1992; Beaglehole 1990). Soy is present in up to 60% of processed foods as a food additive or meat substitute, and soy formula is estimated to constitute around 12% of the infant formula market, a recent decrease from historically higher levels (Barrett 2006). While soy is reported to have antioxidant and anti-cancer properties, the adverse effects of phytoestrogens on reproduction in animals are well established (Ravindranath et al. 2004). Infertility was initially described in 1946 in sheep foraging on red clover, an abundant source of phytoestrogens (Bennets et al 1946). Among the soybean isoflavones, genistein is the most abundant and well characterized (Murphy et al. 2002). Infertility in captive cheetahs was attributed to the high genistein content in their diets and was reversed upon withdrawal of the soy-based diet (Setchell et al. 1987). These examples suggest that phytoestrogens exist in our environment at levels high enough to cause infertility in mammals, and the pervasive use of phytoestrogens in food makes it clear that humans and animals are unavoidably exposed to these compounds.

In addition to estrogenic activities, genistein can regulate the immune response (Masilamani et al. 2012). Genistein regulates human monocyte-derived dendritic cell maturation, secretion of dendritic cell-derived cytokines, and dendritic cell-mediated effector functions in culture (Wei et al. 2012). Interference of immune cell activation by genistein exposure may reflect one mechanism by which genistein causes infertility in mammals.

Classically, anti-inflammatory actions within the immune system are attributed to signaling by endogenous glucocorticoids and synthetic glucocorticoids given therapeutically (Busillo and Cidlowski 2013). Glucocorticoids mediate their biological functions through binding the glucocorticoid receptor (GR), a ligand-dependent transcription factor belonging to the nuclear receptor superfamily (Baxter and Tomkins 1970). Transcriptional antagonism through GR and the estrogen receptor (ER) binding to promoter elements in the glucocorticoid-induced leucine zipper (*GILZ*) gene was recently described in an immortalized human uterine endometrial cell line (Whirledge and Cidlowski 2013). Through binding ER, it is possible that genistein, like estradiol, antagonizes glucocorticoid-induced gene expression in this uterine cell model. Furthermore, overlap in the immune-modulatory functions ascribed to both genistein and glucocorticoids suggest these hormones may target common cellular functions, and exposure to genistein may alter the physiological role of glucocorticoids. In the present study, Ishikawa cells were used as a model to evaluate potential transcriptional antagonism of genistein and glucocorticoids. Special emphasis was given to co-regulated genes, particularly genistein-mediated changes to glucocorticoid-induced genes. Genistein-mediated antagonism of the glucocorticoid-regulated transcriptome in Ishikawa cells may indicate one mechanism by which genistein-exposure may alter the actions of glucocorticoids.

Methods

Reagents

RPMI Medium 1640 (RPMI 1640) was purchased from Invitrogen (Gibco) (Carlsbad, CA). Phenol Red-Free RPMI 1640 was made at The Media Unit of the National Institute of Environmental Health Sciences (Research Triangle Park, NC). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Charcoal stripped heat inactivated FBS was purchased from Hyclone (Logan, UT). Dexamethasone (Dex), Estradiol (E_2), and Mifepristone (RU486) were purchased from Steraloids, Inc (Newport, RI). Fulvestrant (ICI-182,780) and Genistein (Gen) were purchased from Sigma-Aldrich (St Louis, MO). Bisphenol A (BPA) was purchased from Midwest Research Institute (Kansas City, MO). Cycloheximide was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). TaqMan® RT-PCR primer-probes were purchased from Applied Biosystems (Foster City, CA). On-Target Plus® Control Pool (Non-Targeting Pool) and Smart Pool (Human *NR3C1*, *ERα*, and *ERβ*) small interfering RNA (siRNA) were purchased from Thermo Scientific Dharmacon (Waltham, MA).

Culture of human Ishikawa cells

An immortalized uterine human endometrial adenocarcinoma cell line (Ishikawa) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in a standard tissue culture incubator at 37^{o}C, with 95% humidity and 5% carbon dioxide. Ishikawa cells were maintained in RPMI Medium 1640 (Invitrogen (Gibco), Carlsbad, CA) supplemented with 5% FBS. Twenty-four hours prior to cell treatment, media were changed to phenol red-free RPMI 1640 containing 5% charcoal dextran treated (stripped) FBS. Cells were treated with 100

nM Dex prepared in PBS and/or 10 nM E_2, 100 nM Gen or 100 nM BPA prepared in Ethyl Alcohol (EtOH) (The Warner-Graham Company, Cockeysville, MD) for 6 hr. EtOH served as the vehicle control for all studies (100% EtOH diluted to a final concentration of .1% for vehicle). For GR or ER antagonism experiments, 1μM RU486 or 1μM ICI-182,780 prepared in EtOH was added 1 hr prior to agonist treatment. In cyclohexamide experiments, 10 μg/mL of cyclohexamide was added 1 hr before agonist treatment.

Quantitative real-time PCR

RNA isolation and quantitative real-time reverse transcription-PCR (QRT-PCR) were performed as previously described (Whirledge et al. 2013). For additional information see Supplemental Material, RNA Isolation and QRT-PCR. The signal obtained from each gene primer-probe set was normalized to that of the unregulated housekeeping gene peptidylprolyl isomerase B (*PPIB*). Each gene's primer-probe set was evaluated in at least four biological replicates of RNA.

Microarray study

Gene expression analysis was conducted using Agilent Whole Human Genome 4x44 multiplex format oligo arrays (Agilent Technologies, Santa Clara, CA) following the Agilent 1-color microarray-based gene expression analysis protocol and has been previously described (Whirledge et al. 2013). For additional information see Supplemental Material, Microarray Study. A heat map was generated using BioConductor package HeatPlus. Normalized and sample replicates-averaged data of all significant probes were used for calculation of pairwise correlation. Dendrograms of samples (columns) and genes (row) were generated by Hierarchical clustering. Color scale is from 3-fold lower (log2-fold= -1.58) than mean (green) to 3-fold higher (log2-fold= 1.58) than the mean (red). The lists of significant probe sets by treatment were

visually sorted by Venn diagram (http://www.pangloss.com/seidel/Protocols/venn.cgi) and further analyzed by Ingenuity Pathway Analysis (IPA) (version 6.5) (Ingenuity Systems, Redwood City, CA). The average expression value of duplicate identifiers for the same molecule was used in the analyses to eliminate redundancy.

Western blotting analysis

Whole cell lysates were prepared, and western blots were performed as previously described (Whirledge and Cidlowski 2013). For additional information see Supplemental Material, Whole Cell Lysates. Protein was probed with polyclonal anti-GR antibodies (1:1000), monoclonal anti-ER antibodies (1:750; Immunotech, Marseille, France), polyclonal anti-GILZ antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-β-actin antibodies (1:10,000; Millipore, Temecula, CA).

RNA interference

For each biological replicate, cells were plated in 6-well plates at approximately 70% confluency one day prior to transfection. 50nM of each siRNA was transfected into cells with DharmaFECT® 1 transfection reagent (Thermo Scientific) following manufacturer's instructions. The next day each transfected well was split into 4 wells of a 6-well plate for RNA isolation following agonist treatment and 1 10 cm dish for protein isolation. Forty-eight hours following transfection, media was changed to phenol-red free RPMI 1640. After 72 hr of siRNA treatment, cells were induced with 100 nM Dex, 100 nM Gen, or Dex and Gen, and mRNA harvested 6 hr later.

Chromatin immunoprecipitation assays

Chromatin Immunoprecipitation (ChIP) assays were performed using the Magna ChIP A Chromatin Immunoprecipitation kit (Millipore) according to the manufacturer's protocol and as previously described (Whirledge and Cidlowski 2013). For additional information see Supplemental Material, Chromatin Immunoprecipitation Assay. Formalin fixed chromatin was immunoprecipitated overnight with 10 μl of polyclonal GR antibody (Cidlowski et al. 1990), 10 μl of monoclonal ER antibody, or 2 μl of IgG (Millipore, Billerica, MA, USA). PCR analysis of the GR response element (GRE), transcriptional start site (TSS), and control sequences utilized specific primers (Supplemental Material, Table S1).

Statistical analysis

Data are presented as means ±SEM. Statistical significance was determined by ANOVA with Tukey's post-hoc analysis. Statistical significance is reported as either * $p< 0.05$ or ** $p< 0.01$.

Results

Estradiol regulates almost 3,000 genes in immortalized human uterine endometrial adenocarcinoma cells, and the spectrum of regulation largely overlaps with those genes regulated by glucocorticoids (Whirledge and Cidlowski 2013; Whirledge et al. 2013). Interestingly, estradiol co-administered with dexamethasone in these cells alters and antagonizes glucocorticoid-induced gene expression over a range of concentrations (0.01 nM-10 nM E_2) (Whirledge and Cidlowski 2013). To determine whether environmental estrogens are also able to antagonize glucocorticoid-induced gene expression, *GILZ* mRNA was quantified by QRT-PCR in Ishikawa cells after stimulation with Dex, E_2, Gen, BPA, or a combination of Dex with estrogen. At a timepoint where Dex significantly upregulates *GILZ* mRNA, E_2 and genistein, but

not BPA, antagonized the effect of Dex (Figure 1A). Antagonism by genistein was significant at 6 and 24 hr (Figure 1A). To determine the physiological relevance of genistein antagonism, the range of concentrations at which genistein can antagonize glucocorticoid-induced gene expression (*GILZ*) was evaluated in cells treated with 1 to 1000 nM Gen and 100 nM Dex (Figure 1A). At all genistein concentrations evaluated, Dex-induced *GILZ* gene expression was significantly repressed. At the concentration of 100 nM genistein, repression of glucocorticoid-induced *GILZ* expression was maximal, and this concentration was chosen for all subsequent experiments.

Microarray analysis was performed following treatment with genistein or estradiol to evaluate the transcriptional response to genistein in Ishikawa cells and determine the genome wide common and unique targets of estradiol and genistein. Surprisingly, less than a third of the genes significantly regulated by genistein were in common with estradiol treatment, 268 out of 932 gene probes (Figure 1B). A majority of the genes common to estradiol and genistein treatment were regulated in a similar manner. However, some genes were identified as antagonistically regulated or anti-correlated, suggesting estradiol and genistein exposure may not result in the same transcriptional profile. Examples of the patterns identified are shown in Figure 1C. Furthermore, network analysis utilizing the Ingenuity Pathway Analysis (IPA) analysis indicates genes regulated by genistein represent vastly different primary networks than those regulated by estradiol (Supplemental Material, Table S2). Genistein has been reported to preferentially bind ERβ, as compared to the affinity of E_2 for ERα and β (Manas et al. 2004). To evaluate whether preferential ligand binding is responsible for differences in gene expression profiles, mRNA of two genes with E_2- and genistein-specific expression patterns were quantified following ERα or

ERβ knockdown. Cells transfected with siRNA against ERα or ERβ, but not the non-targeting control pool, demonstrated significantly less ERα and ERβ mRNA, respectively (Figure 1D). Expression of Angiotensinogen (*AGT*) and Natriuretic Peptide Type C (*NPPC*) mRNA was evaluated in transfected cells following treatment for 6 hr with vehicle, E_2, or Gen. *AGT* and *NPPC* were significantly induced by E_2 and genistein (Figure 1D). Knockdown of ERα but not ERβ abolished E_2 induction of *AGT* and *NPPC*, indicating ERα likely mediates E_2 regulation of these genes. Genistein regulation of AGT also required ERα but not ERβ (Figure1D). However, in the presence of *ERα* or *ERβ* siRNA, genistein induction of *NPPC* was significantly less, suggesting genistein regulation of *NPPC* requires both ERα and ERβ.

Dexamethasone and estradiol co-regulate global gene expression in Ishikawa cells (Whirledge et al. 2013). In light of the unique gene regulation by genistein, the gene lists of Dex + E_2 were compared to that of Dex + Gen. Interestingly, the combination of glucocorticoids and genistein regulates a set of genes unique from those regulated by glucocorticoids and estradiol (Figure 1E). Though 1016 genes are common to Dex + E_2 and Dex + Gen treatment groups, a significant number of these commonly regulated genes are anti-correlated or demonstrate antagonistic regulation when comparing the two treatment paradigms. A representative gene demonstrating all patterns of direction discovered is provided (Figure 1F). Furthermore, IPA analysis indicates that genes regulated by glucocorticoids and genistein represent distinct gene networks from those regulated by glucocorticoids and estradiol (Supplemental Material, Table S3). Those deviating gene networks indicate genistein regulates a unique, and perhaps divergent, transcriptome in the presence of glucocorticoids in Ishikawa cells.

Based on the unique transcriptional profile of genistein, microarray analysis was performed to identify genes regulated by both dexamethasone and genistein in Ishikawa cells. Comparison of significantly regulated probes identified 5,893 genes regulated by Dex, Gen, or a combination of Dex + Gen. Gene profiles are shown as a heat map representing raw data (Figure 2A). Venn diagram analysis compared the gene lists to identify those genes that are common and unique to each of the three treatment groups (Figure 2A). Genistein treatment regulates 932 genes, Dex treatment regulates 1633 genes, and 3328 genes are regulated only by the combination of Dex and Gen. Unexpectedly, two thirds of the Dex + Gen genes are regulated only in the presence of Dex and Gen and not by Dex or Gen alone, representing previously unidentified molecular gene targets.

Venn diagram analysis indicates several genes are targets of both glucocorticoids and genistein. The top five induced and repressed genes by treatment are listed (Table 1). Several genes are regulated in all treatment groups, although expression levels differ by treatment. The expression chart for each gene illustrates the variation in regulation by treatment (each bar represents Dex, Gen, Dex + Gen, from left to right respectively) (Figure 2B). To examine the extent of transcriptional remodeling for Dex and Gen regulated genes, co-regulated genes from the overlapping gene lists were classified as: induced by both Dex and Gen, repressed by both Dex and Gen, genes with opposing direction of regulation (anti-correlated), or antagonistically regulated, and a representative gene demonstrating these patterns is provided (Figure 2C). Almost 7% of commonly regulated genes (22 genes) were found to be anti-correlated (Supplemental Material, Table S4), and 25 genes were found to be antagonistically regulated. Select genes representing different patterns of regulation were independently validated though

the QRT-PCR analysis of four independent biological replicates not included in the initial microarray analysis (Supplemental Material, Figure S1). Period circadian clock 1 (*PER1*) represented a Dex-induced gene and validated as such. Leukemia Inhibitory Factor (*LIF*) was validated as a gene repressed in all treatment groups. *NPPC* and Carbonic Anhydrase 12 (*CA12*) were validated as Gen-induced genes, and interestingly, Dex antagonizes Gen-induction for both genes. Guanosine monophosphate reductase (*GMPR*) was identified as a Dex-induced gene antagonized by Gen co-treatment and validated as such. Microseminoprotein, beta (*MSMB*) demonstrates synergistic regulation, where the induction by Dex + Gen is greater than Dex or Gen alone.

To determine whether GR and ER are required for the transcriptional regulation by Dex and Gen in common gene targets, cells were pre-treated for 1 hr with either the GR antagonist RU486 or the ER antagonist ICI-182,780, and gene expression of select co-regulated genes was analyzed 6hr following treatment with Dex, Gen, or Dex + Gen (Figure 3). To confirm that ICI-182,780 exposure, which competitively binds, down-regulates and degrades both ERα and ERβ, resulted in lower ER expression, western blot analysis was performed on whole cell lysates from cells treated for 7 hr with ICI-182,780 (Figure 3A). ICI-182,780 significantly decreased ERα protein levels. RU486 did not significantly alter GR or ER protein levels (data not shown), but the absence of GILZ mRNA induction following Dex treatment indicated that GR-actions were blocked and the efficacy of this antagonist. In the microarray and by independent validation, *CA12* mRNA was found to be induced by Gen and antagonistically regulated by Dex. Induction of *CA12* mRNA is blocked when cells were pre-treated with the ER antagonist ICI-182,780

(Figure 3B). Dex antagonism of Gen-mediated *CA12* induction in the Dex + Gen treated cells is fully relieved by pre-treatment with the GR antagonist RU486, suggesting both receptors are likely involved in co-regulation of *CA12*. Antagonistic regulation of Left-Right Determination Factor 1 (*LEFTY1*) by dexamethasone and estradiol in Ishikawa cells was previously shown to be mediated by GR and ER (Whirledge et al. 2013). Similar to estradiol, genistein antagonizes GR-mediated dexamethasone-induced *LEFTY1* expression (Figure 3B). Genistein antagonism is blocked by the ER antagonist ICI-182,780, and RU486 blocks Dex-induction of *LEFTY1* mRNA expression, indicating co-regulation of *LEFTY1* gene expression by glucocorticoids and genistein requires GR and ER. In human uterine endometrial adenocarcinoma cells, as well as in the mouse uterus, dexamethasone and estradiol regulate the expression of the *GILZ* gene through GR and ERα (Whirledge and Cidlowski 2013). Dex-induced expression of *GILZ* is also antagonized by Gen treatment, and the ER antagonist ICI-182,780 abrogates this antagonism (Figure 3B).

Analysis of gene expression in the presence of GR and ER inhibitors indicates these receptors are required for the antagonistic regulation of *CA12*, *LEFTY1*, and *GILZ* by Dex and Gen. To evaluate the requirement of GR and ERα more specifically, cells were transfected with siRNA against *GR* and *ERα* prior to agonist treatment. Transfection with siRNA against *GR* and *ERα*, but not the non-targeting control pool, was able to significantly reduce the expression GR and ERα protein without affecting levels of the house keeping gene β-actin (Figure 3C). Cells transfected with NTC pool, *GR*, or *ERα* siRNA and treated for 6 hr with vehicle, Dex, Gen, or Dex and Gen were examined for *CA12*, *LEFTY1*, and *GILZ* mRNA expression by QRT-PCR (Figure 3D). Knockdown of GR expression by siRNA abrogated the ability of Dex to repress Gen-induced *CA12* mRNA expression. Furthermore, ERα knockdown eliminated induction of

CA12 by genistein, confirming the necessity of GR and ERα in the co-regulation of *CA12*. For both of the glucocorticoid-induced genes, repression of GR by siRNA attenuated *LEFTY1* and *GILZ* induction by Dex. ERα knockdown by siRNA relieved the antagonism brought about by genistein treatment. Interestingly, induction of *GILZ* mRNA by Dex increased in cells transfected with *ERα* siRNA.

Based on the mechanism discovered for the co-regulation of *GILZ* by glucocorticoids and estradiol, transcriptional regulation of *GILZ* was more closely examined to determine if genistein and estradiol share a common mechanism of antagonistic regulation. To understand the mechanism by which genistein antagonizes glucocorticoid-induced *GILZ* expression, treated cells were evaluated for evidence of indirect or direct regulation by Gen. Cells were administered cycloheximide 1 hr prior to hormone treatment, and *GILZ* mRNA levels were quantified 6 hr after treatment (Supplemental Material, Figure S2A). Cycloheximide pretreatment did not alter the ability of Gen to repress Dex-induced *GILZ* mRNA expression. Nascent *GILZ* RNA expression in response to Dex and Dex + Gen treatment was examined to determine whether genistein antagonism of glucocorticoid-induced gene expression is a function of impeding transcriptional initiation (Supplemental Material, Figure S2B). Co-treatment with Dex and Gen represses mature and nascent RNA transcripts. These data indicate genistein directly regulates glucocorticoid-induced *GILZ* expression at the level of transcription. Analysis of GILZ protein expression following 24 hr treatment indicates changes in mRNA message levels are translated into differences in protein expression (Supplemental Material, Figure S2C).

A narrow *in silico* promoter analysis was performed on genes identified as anti-correlated or antagonistically regulated to identify response elements for GR and ER. This would suggest one

mechanism by which these genes are regulated by glucocorticoids and genistein is through their respective receptors binding promoter elements. The open-access JASPAR database of matrix-based transcription factor binding profiles was utilized to search 3,000 bp upstream and 500 bp downstream of the transcriptional start site of each gene (Bryne et al. 2008). A total of 319 genes were determined to be anti-correlated or antagonistically regulated and 233 contained an annotated promoter. Genes with an annotated promoter were analyzed for the inclusion of a GRE, nGRE (sequence I and II), or ERE sequence and compared to 100 genes found in the microarray to not be significantly regulated by Dex or Gen to determine enrichment of response elements (Figure 4A). Compared to unregulated genes, antagonized genes demonstrate an enrichment of 129% for GREs, 142% for nGRE I, 274% for nGRE II, and 188% for the ERE sequence. The enhanced presence of GR and ER response elements in co-regulated genes may signify the ability of these steroid receptors to directly regulate the expression of target genes.

Given that ERα is required for the Gen-mediated antagonism of GR-induced *GILZ* expression, ChIP assays were performed on human genomic DNA from Ishikawa cells treated for 1 hr with vehicle, Dex, Gen, or Dex and Gen to examine the recruitment of ERα to the *GILZ* promoter *in vitro* (Figure 4B). Occupancy of GR and ERα at the GRE located at position -1919 to -1794 in the promoter and the transcriptional start site of *GILZ* was described following Dex and E_2 stimulation in human endometrial cells (Whirledge and Cidlowski 2013). In a mechanism similar to the estradiol-like antagonism of Dex-induced *GILZ* gene expression, both GR and ERα are recruited to the GRE located at -1919 to -1794 and the transcriptional start site of *GILZ* in the presence of Dex or Gen, respectively (Figure 4B). In the presence of Dex and Gen, GR association with the chromatin at the GRE -1919 to -1794 and the transcriptional start site is

reduced, offering a potential mechanism by which glucocorticoid-mediated up-regulation of *GILZ* mRNA antagonized by the presence of genistein and glucocorticoids. These data suggest that estradiol share both common and divergent mechanisms of gene regulation and interactions with other transcription factors. Recruitment of GR to a Nuclear Factor κB (NFκB) site in Interleukin 8 (*IL-8*) and ERα to an ERE in Trefoil Factor 1 (*TFF1*) served as a controls (Figure 4C). Under the same concentrations of Dex that induced recruitment of GR to the promoter of GILZ, the occupancy of GR near a NFκB site in *IL-8* is significantly enhanced 1 hr following treatment compared to vehicle. ERα occupancy of the evaluated ERE in the *TFF1* promoter is significantly increased at the same concentration of Gen used to induce ERα recruitment to the GILZ promoter following 1 hr treatment.

Discussion

Reported pathologies of the female reproductive tract in animal models attributed to genistein exposure have raised concerns regarding the widespread inclusion of soy in commercially processed food and as a large constituent of the infant formula market (Barrett 2006). The present study provides important evidence that genistein exposure induces a significantly different transcriptional response than estradiol in Ishikawa cells. We previously showed that the endogenous estrogen estradiol regulates GR-mediated transcription in Ishikawa cells *in vitro* and in the mouse uterus (Whirledge and Cidlowski 2013; Whirledge et al. 2013). We now demonstrate through whole genome microarray analysis that the response to genistein alone and in combination with dexamethasone is significantly different from that of estradiol or estradiol with dexamethasone under the same conditions. In fact, a majority of the gene probes regulated by genistein are not in common with estradiol. Genistein-regulated genes represent distinct gene

networks, suggesting that genistein regulates distinct biological pathways in these immortalized endometrial cells. Whether this represents the actual physiology of normal endometrium will require additional studies in mouse and human cell model systems. In the presence of dexamethasone, genistein regulates unique genes independently of estradiol. These differences may reflect altered ERα and ERβ utilization by genistein, as compared to estradiol, or genistein regulation of other steroid receptors' expression. Genistein also demonstrates potent tyrosine kinase inhibitor activities (Akiyama et al. 1987). However, these effects occur at much higher concentrations than employed in this study (10-100 μM), and when the results of the receptor knockdown experiments are considered, the glucocorticoid antagonist properties of genistein appear to be mediated through ERα for those genes studied (Barnes et al. 2000).

The interactions between steroid receptors are poorly understood, including the mechanisms by which GR and ERα regulate gene transcription. Altered GR and ERα recruitment to the promoter of the glucocorticoid-induced gene *GILZ* mediates the antagonistic regulation by dexamethasone and estradiol (Whirledge and Cidlowski 2013). However, the mechanism of co-regulation by GR and ER has not yet been discovered for those newly identified common targets of dexamethasone and genistein. Evaluating global transcription factor binding in breast cancer cells indicates GR and ER can mediate genomic cross-talk by regulating each other's binding at recognition sites. This allows rapid reprogramming of the chromatin structure and targeting of novel genes following the co-activation of both receptors (Miranda et al. 2013). This model of transcription factor interplay is likely responsible for the regulation of those common gene targets, as well as genes only found to be induced or repressed in the presence of dexamethasone and genistein. Interestingly, less than one-third of dexamethasone and genistein co-regulated

genes are in common with those regulated by dexamethasone and estradiol. In the model of molecular interplay between GR and ER, this may indicate that genistein bound ER is recruited to different recognition sites or results in alternative cofactor recruitment when compared to ER bound to the endogenous ligand (Chang et al. 2008). In fact, altered genome-wide ERα binding following genistein treatment is evident in endometrial cancer cells and suggests one mechanism by which estradiol and genistein treatments result in different patterns of gene expression (Gertz et al. 2012).

In order to understand how glucocorticoids and genistein regulate common genes, three novel targets were evaluated in greater detail. Our study is the first to report transcriptional regulation of *CA12*, encoding a membrane-associated protein responsible for the acidification of the microenvironment, by genistein in an immortalized human uterine cell line. In the female reproductive tract, CA12 is localized to the endometrium in both the mouse and human uterus, where maintenance of appropriate pH levels is critical to the fertilization process (Karhumaa et al. 2000; Hynninen et al. 2004). CA12 is found at high levels in the proliferative endometrium, a phase characterized by high levels of estradiol, suggesting aberrant induction by environmental estrogens may disrupt the precision of timing necessary for fertilization (Ivanov et al. 2001). Although one previous report indicates CA12 is a target of GR signaling, glucocorticoids are able to accelerate the enzymatic activity of carbonic anhydrases (Endroczi et al. 1994; Donn et al. 2007). The ability of endogenous cortisol, which also varies with the menstrual cycle, to antagonize genistein-induced *CA12* gene expression is unknown. Similarly, the degree to which aberrant CA12 expression compromises fertility in not appreciated (Nepomnaschy et al. 2011).

Transcriptional regulation of *LEFTY1* and *GILZ* by dexamethasone and the environmental estrogen genistein has not been previously reported (Whirledge and Cidlowski 2013; Whirledge et al. 2013). The *in vivo* significance of glucocorticoid regulation of *LEFTY1* and *GILZ* in the uterus is not clear, but both LEFTY1 and GILZ regulate important biological functions. LEFTY1 has been shown to play an important role in uterine decidualization and embryo implantation (Tabibzadeh 2011). Furthermore, LEFTY1 is temporally expressed in the endometrium of fertile women (Kothapalli et al. 1997). Alterations to the precise timing of GILZ expression may also adversely affect uterine biology, especially the immuno-modulatory effects of GILZ. GILZ is an important mediator of T lymphocyte activation, function, and cell death, immune cells essential to implantation and early stages of pregnancy (Ayroldi and Riccardi 2009; Nevers et al. 2011). Estradiol and genistein share a similar mechanism by which glucocorticoid-induced *GILZ* expression is antagonized. However, dietary genistein exposure does not follow the natural patterns of cyclic estradiol and could result in aberrant GILZ expression. The consequences of this are unknown but have the potential to directly affect immune tolerance within the uterus.

Conclusion

Although genistein has been found to be clinically beneficial in relation to cardiovascular disease and cancer, research indicates that consuming environmentally relevant doses of genistein has adverse effects on the female reproductive tract in mammals. Modulation of ER activity is partially responsible for the uterotrophic effects of genistein, and accordingly, we observed some overlap between genistein and estradiol regulated genes in Ishikawa cells; however, two-thirds of genistein-regulated genes are unique to this treatment. Furthermore, in the presence of genistein, dexamethasone exposure results in a divergent pattern of gene regulation. Our findings

demonstrate one mechanism by which genistein may directly regulate GR-mediate gene expression and represent an important *in vitro* model to discover the molecular actions of genistein. In addition to those select genes studied, co-regulated genes identified by genomic analysis may provide exciting molecular targets with potential biological insight.

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Table 1. Fold change of the top five induced and repressed genes by treatment.

Gene	Fold change
Dex	
PRR16	11.71
GMPR	10.39
PNMT	9.46
TDRD9	8.84
PER1	8.60
FRMD4A	-9.23
TMEM191B	-15.05
NEUROG1	-16.06
ELF5	-19.49
ZNF775	-25.45
Gen	
CACNA1I	20.67
MSMB	19.54
NPPC	9.39
ALPPL2	9.24
CA12	8.95
FOXB1	-23.44
ATN1	-28.07
SYNPO	-35.99
GRIN1	-37.20
ZNF775	-40.56
Dex + Gen	
MSMB	30.26
MICALCL	16.38
CACNA1I	15.48
CAMP	12.98
DHRS3	11.49
SP5	-17.21
NEUROG1	-17.68
TMEM191B	-18.02
ELF5	-39.80
ZNF775	-45.33

Figure Legends

Figure 1. Genistein regulates gene expression in Ishikawa cells independently of estradiol. A) Expression of Glucocorticoid-Induced Leucine Zipper (*GILZ*) mRNA was measured by QRT-PCR following 6 hr treatment with vehicle, 100 nM Dex, 10 nM E_2, 100 nM Gen, 100 nM BPA, or Dex + E_2, Dex + Gen, or Dex + BPA (concentrations used in combination same as independent treatment). *GILZ* mRNA was also measured at 2, 6, and 24 hr after vehicle, Dex, Gen, or Dex + Gen treatment. Expression of *GILZ* was measured by QRT-PCR in cells treated for 6 hr with vehicle, Gen at 0 nM – 1000 nM, or 1 nM – 1000 nM Gen and 100 nM Dex. Values were normalized to the housekeeping gene Cyclophilin B (*PPIB*). Bar graphs show mean ± SEM of four biological replicates. * denotes groups with statistically different means at $p < 0.05$ (** $p < 0.01$). B) mRNA isolated from 3 biological replicates treated with E_2 or Gen for 6 hr were analyzed by the Agilent Whole Human Genome 4x44 multiplex format oligo array for gene expression. The number of probes statistically different ($p < 0.01$) between treatment groups were sorted by Venn diagram. C) E_2 and Gen co-regulated genes were separated by direction of regulation. One gene that represents each pattern of regulation is displayed (induced, repressed, anti-correlated, and antagonized, from left to right). D) Cells transfected with non-targeting control pool (NTC), *ERα*, or *ERβ* siRNA were assessed for the extent of knockdown by QRT-PCR (n=4). Transfected cells were treated for 6 hr with vehicle, E_2, or Gen, and mRNA of Angiotensinogen (*AGT*) and Natriuretic Peptide Type C (*NPPC*) were measured. Values were normalized to the housekeeping gene Cyclophilin B (*PPIB*). * denotes groups with statistically different means at $p < 0.05$ (** $p < 0.01$). E) Microarray profile showing comparison of Dex + E_2 and Dex + Gen treatment, as sorted by Venn diagram. F) Dex + E_2 and Dex + Gen co-regulated

genes were separated by direction of regulation. One gene that represents each pattern of regulation is displayed (induced, repressed, anti-correlated, and antagonized, from left to right).

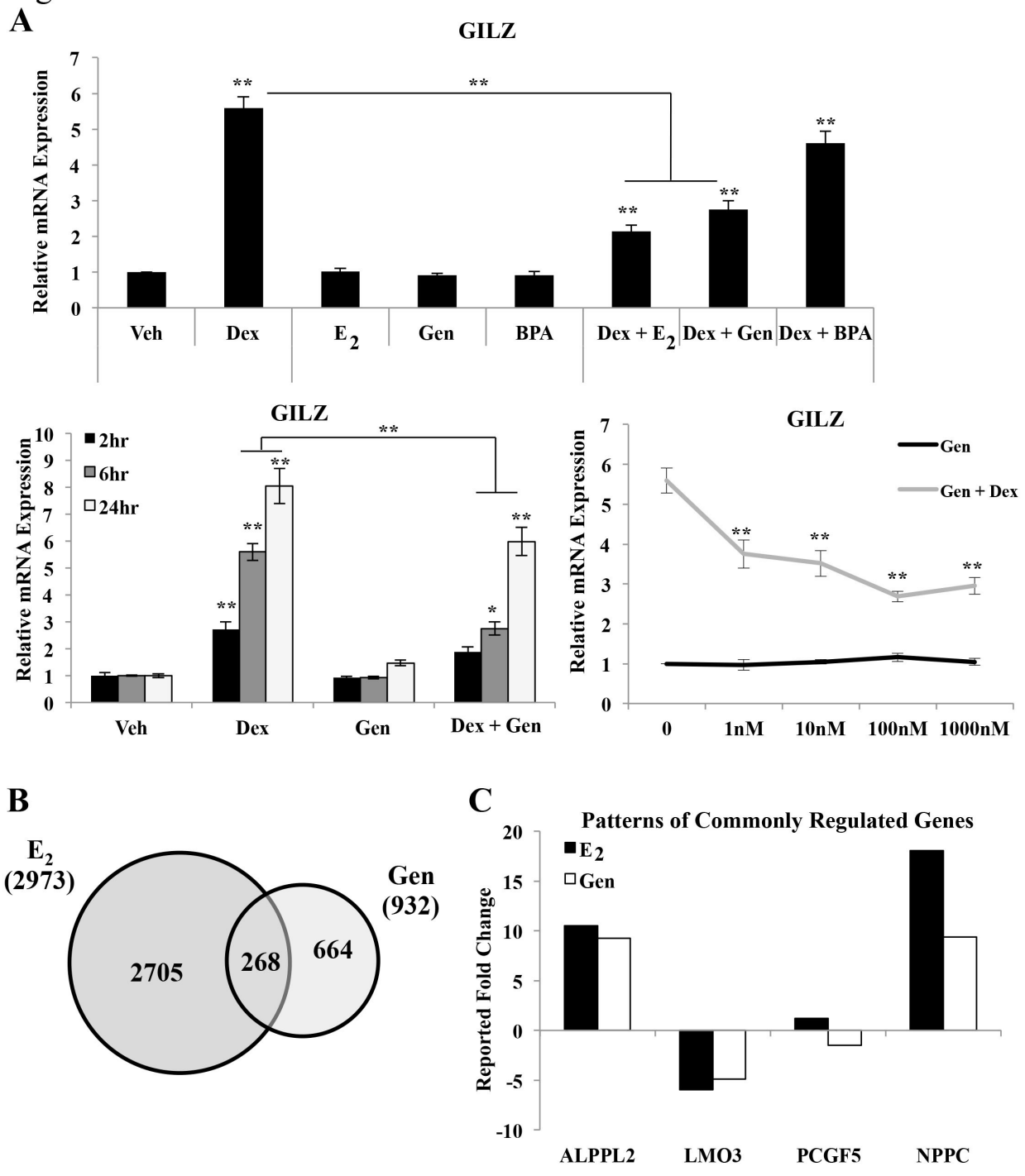
Figure 2. Microarray analysis reveals common and unique targets of genistein and dexamethasone. A) The number of probes regulated by Dex, Gen, and D + G from three biological replicates are organized as either induced (red) or repressed (green) according to treatment group. The number of probes statistically different ($p < 0.01$) between treatment groups were sorted by Venn diagram. B) The top five induced and repressed co-regulated by Dex, Gen, and D + G are organized by treatment group. The expression chart illustrates the relative fold change from vehicle for the Dex, Gen, and D + G treatment group, starting from left to right. C) Dex, Gen, and D + G co-regulated genes were separated by direction of regulation. A representative gene for all discovered patterns of regulation is displayed (induced, repressed, anti-correlated, and antagonized, from left to right).

Figure 3. The Glucocorticoid and Estrogen Receptor are required for transcriptional antagonism of three commonly regulated genes. A) Cells treated 7 hr with the ER antagonist ICI 182,780 or the GR antagonist RU486 were assayed for ERα and GR protein levels by Western blot. Bar graphs show mean ± SEM of four biological replicates. (** $p < 0.01$) B) Cells were pretreated 1 hr with ICI 182,780 or RU486 and then vehicle, Dex, Gen, or Dex + Gen was added for 6 hr. mRNA expression was determined for Carbonic Anhydrase 12 (*CA12*), Left-Right Determination Factor 1 (*LEFTY1*), and Glucocorticoid-induced Leucine Zipper (*GILZ*). (n=4; * $p < 0.05$, ** $p < 0.01$) C) Cells transfected with ERα siRNA or non-targeting control (NTC) were assessed for the extent of knockdown compared to NTC by Western blotting. (n=4; ** $p < 0.01$) D) Cells transfected with NTC, *GR* siRNA, or *ERα* siRNA were treated for 6 hr with vehicle,

Dex and/or Gen, and mRNA expression of *CA12*, *LEFTY1*, and *GILZ* was evaluated. All mRNA values were normalized to the housekeeping gene Cyclophilin B (*PPIB*) and protein values were normalized to β-actin then compared with vehicle-treated cells. (n=4; * $p < 0.05$, ** $p < 0.01$)

Figure 4. Enhanced promoter recruitment represents one mechanism of glucocorticoid and genistein transcriptional regulation. A) The JASPAR CORE Vertebrata server was used to search a 3500 bp region around the transcriptional start site of annotated genes. Antagonistically regulated genes were compared to 100 genes from the microarray platform that were not significantly regulated by Dex or Gen. B) Recruitment of GR and ERα to the GRE at position -1919 **-** -1794 and to the transcriptional start site (TSS) was assessed by Chromatin Immunoprecipitation (ChIP) assay following treatment with vehicle, Dex, Gen, or Dex and Gen for 1 hr. Enrichment of the sequences containing the GRE and TSS promoter region was measured by QRT-PCR. Bar graphs show mean ± SEM of at least five biological replicates. (** $p < 0.01$) C) Recruitment of GR to an NFκB site in *IL-8* and ERα to an ERE in *TFF1* was analyzed by ChIP and quantified by QRT-PCR for control. All values are normalized to input and set relative to vehicle IgG. (n=5; ** $p < 0.01$)

Figure 1


A
GILZ
Relative mRNA Expression
Veh
Dex
E2
Gen
BPA
Dex + E2
Dex + Gen
Dex + BPA
GILZ
2hr
6hr
24hr
Relative mRNA Expression
Veh
Dex
Gen
Dex + Gen
GILZ
Gen
Gen + Dex
Relative mRNA Expression
0
1nM
10nM
100nM
1000nM
B
E2 (2973)
Gen (932)
2705
268
664
C
Patterns of Commonly Regulated Genes
E2
Gen
Reported Fold Change
ALPPL2
LMO3
PCGF5
NPPC

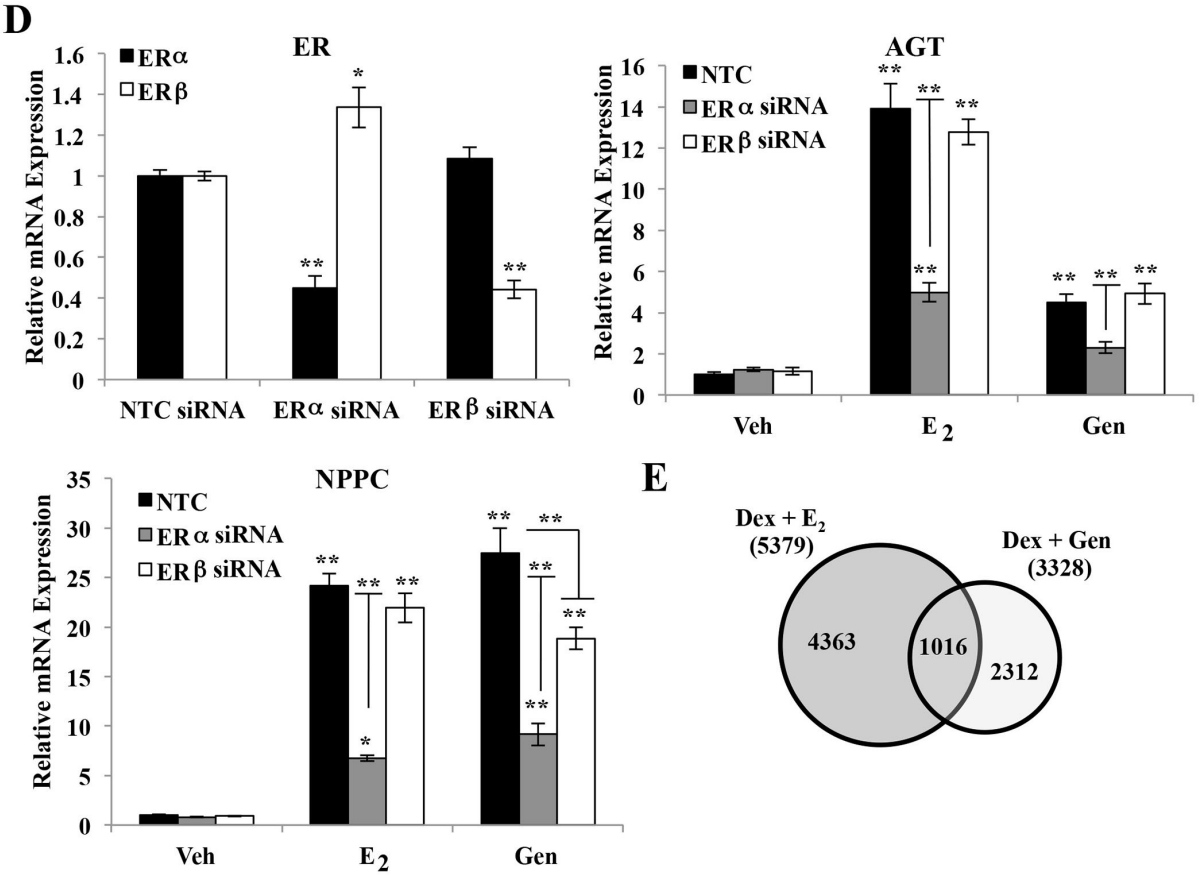
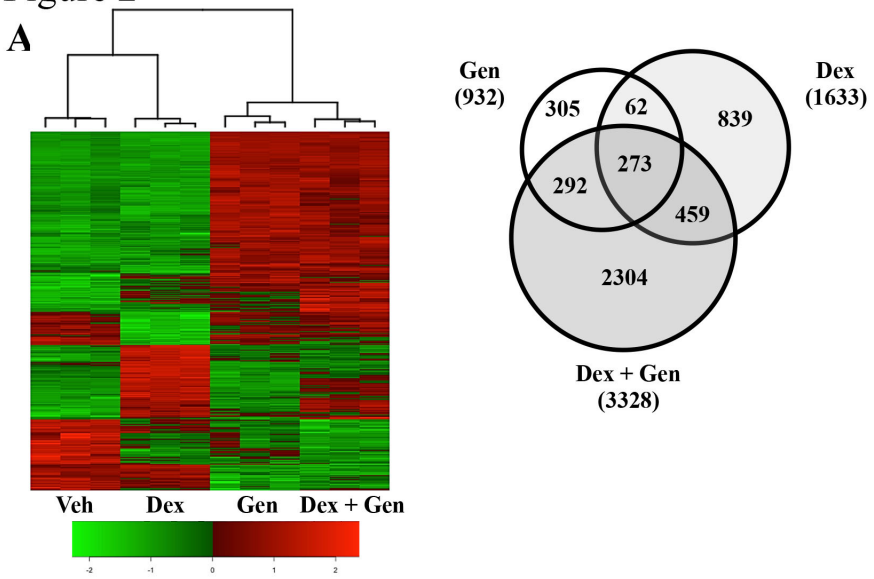

D
ER
ERα
ERβ
Relative mRNA Expression
NTC siRNA
ERα siRNA
ERβ siRNA
AGT
NTC
ERα siRNA
ERβ siRNA
Veh
E2
Gen
NPPC
E
Dex + E2 (5379)
Dex + Gen (3328)
4363
1016
2312
F
Patterns of Commonly Regulated Genes
Dex + E2
Dex + Gen
Reported Fold Change
CAMP
CYP1A1
IER5
AGT

Figure 2



B

Dex

Molecules	Fold-Change	Exp. Chart
CDH1	↑1.212	
ORMDL3	↑1.211	
ARHGEF25	↑1.178	
TIAM1	↑1.105	
IGF2	↑1.105	
ZNF775	↓-25.459	
ELF5	↓-19.490	
UHRF1	↓-14.612	
SSU72	↓-13.086	
ATN1	↓-7.200	

Gen

Molecules	Fold-Change	Exp. Chart
NPPC	↑9.395	
ALPPL2	↑9.241	
CA12	↑8.954	
MLPH	↑8.354	
GDPD5	↑4.725	
ZNF775	↓-40.569	
UHRF1	↓-37.994	
SYNPO	↓-35.991	
SSU72	↓-29.771	
ATN1	↓-28.077	

Dex + Gen

Molecules	Fold-Change	Exp. Chart
MLPH	↑9.786	
CA12	↑8.653	
ALPPL2	↑7.407	
NPPC	↑6.367	
GDPD5	↑4.559	
ELF5	↓-39.809	
SSU72	↓-17.099	
UHRF1	↓-13.291	
SYNPO	↓-10.010	
FAM178B	↓-9.994	

C

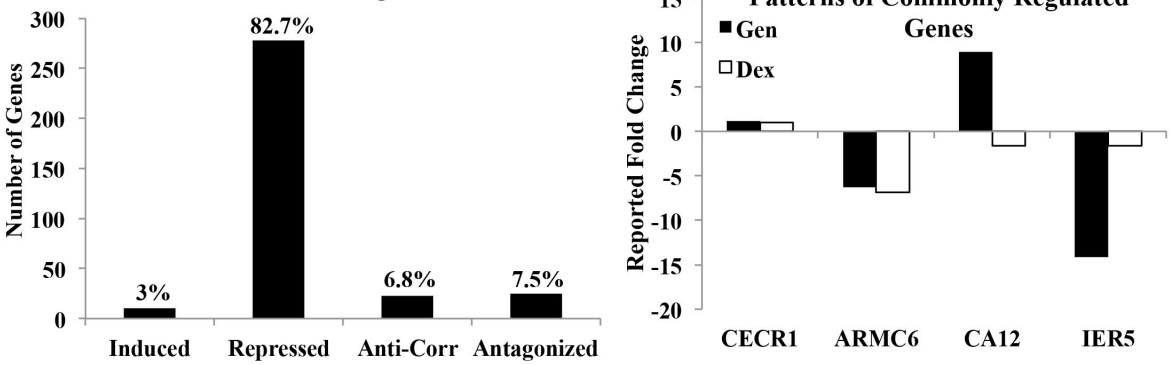
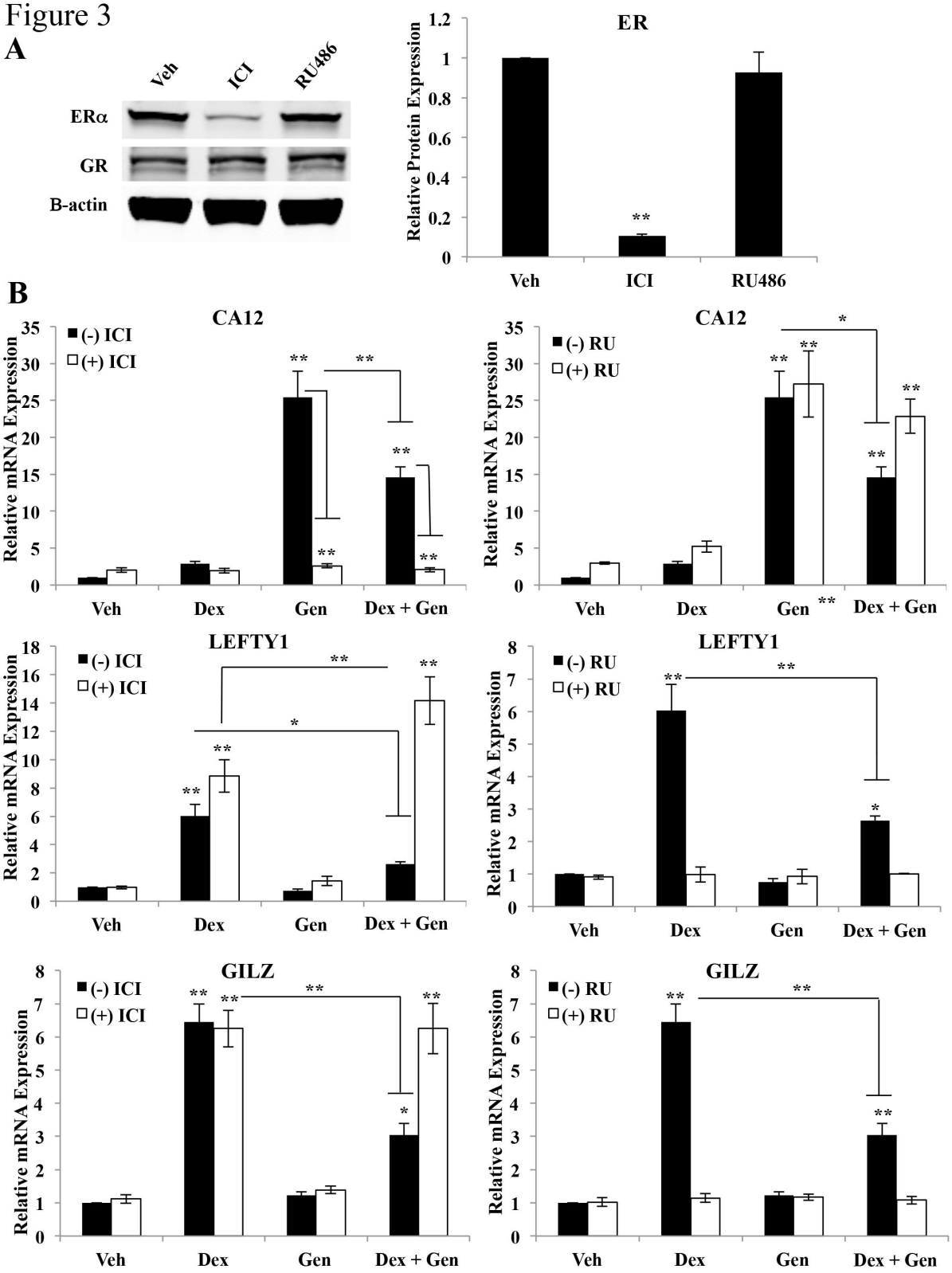


Figure 3


A
Veh
ICI
RU486
ERα
GR
B-actin
ER
Relative Protein Expression
Veh
ICI
RU486
B
CA12
(-) ICI
(+) ICI
Relative mRNA Expression
Veh
Dex
Gen
Dex + Gen
CA12
(-) RU
(+) RU
LEFTY1
(-) ICI
(+) ICI
LEFTY1
(-) RU
(+) RU
GILZ
(-) ICI
(+) ICI
GILZ
(-) RU
(+) RU

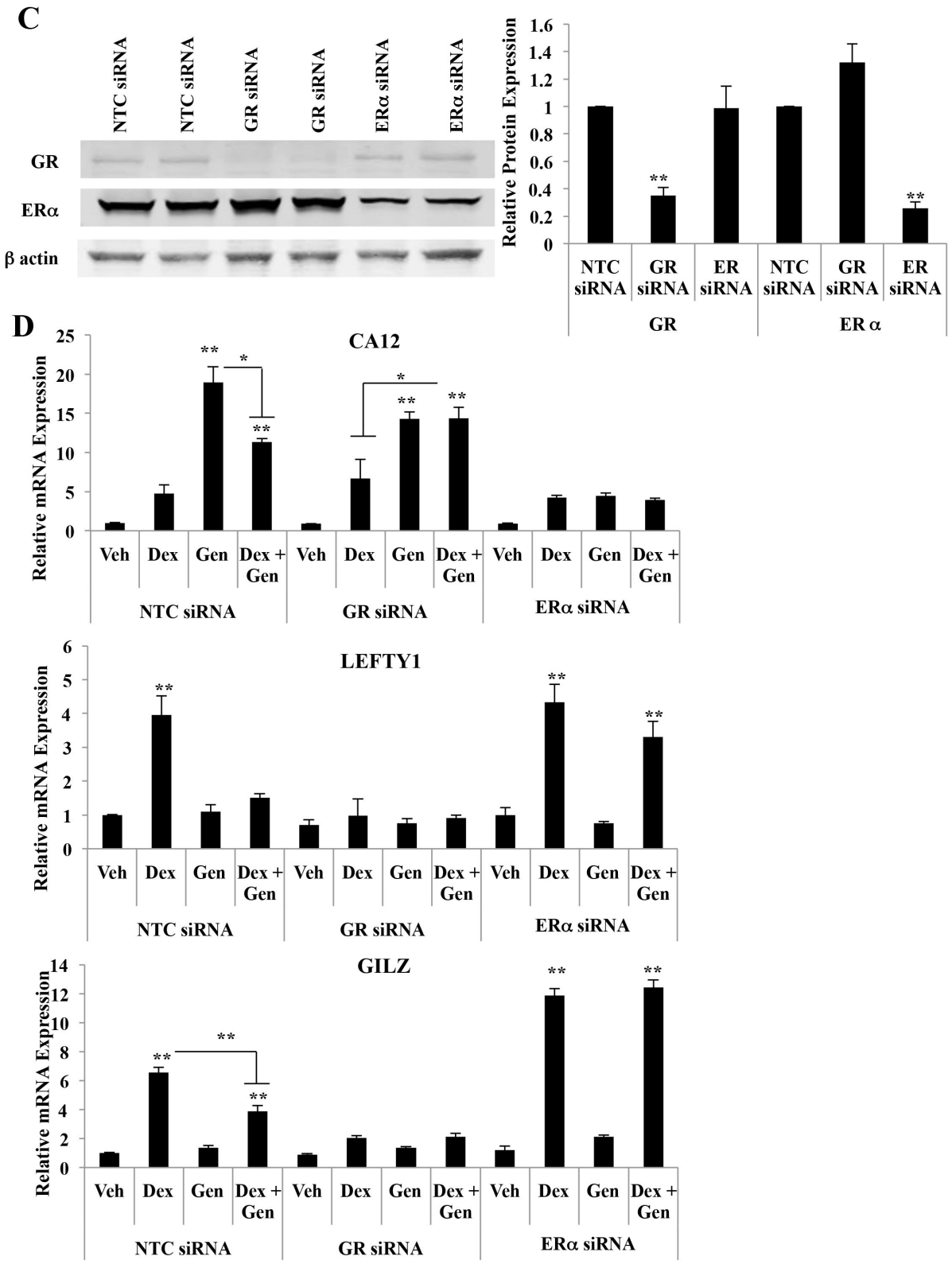

C
NTC siRNA
NTC siRNA
GR siRNA
GR siRNA
ERα siRNA
ERα siRNA
GR
ERα
β actin
Relative Protein Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
NTC siRNA
GR siRNA
ER siRNA
NTC siRNA
GR siRNA
ER siRNA
GR
ER α
D
CA12
Relative mRNA Expression
25
20
15
10
5
0
**
*
**
*
**
**
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
NTC siRNA
GR siRNA
ERα siRNA
LEFTY1
Relative mRNA Expression
6
5
4
3
2
1
0
**
**
**
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
NTC siRNA
GR siRNA
ERα siRNA
GILZ
Relative mRNA Expression
14
12
10
8
6
4
2
0
**
**
**
**
**
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
Veh
Dex
Gen
Dex + Gen
NTC siRNA
GR siRNA
ERα siRNA

Figure 4

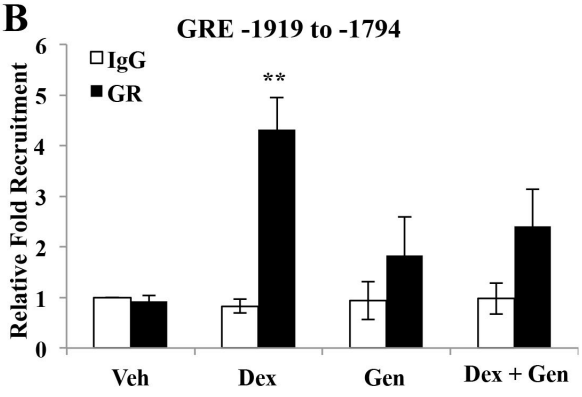
A

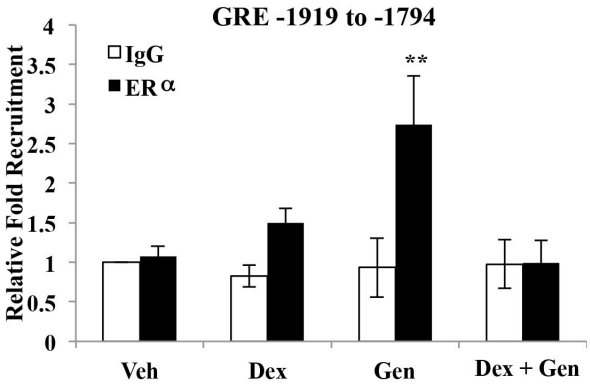
Promoter Analysis

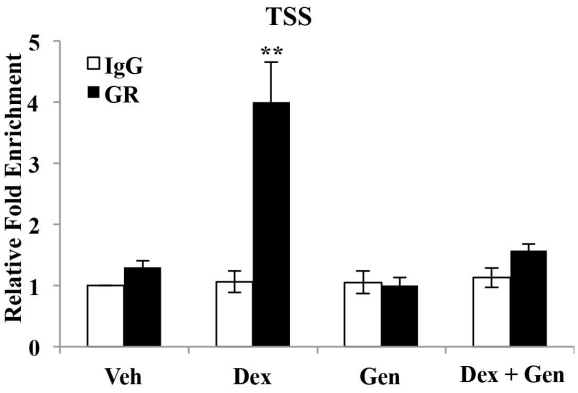
	Unregulated	Regulated	Enrichment
GRE	21%	27%	129%
nGRE I	6%	8.5%	142%
nGRE II	5%	13.7%	274%
ERE	5%	9.4%	188%

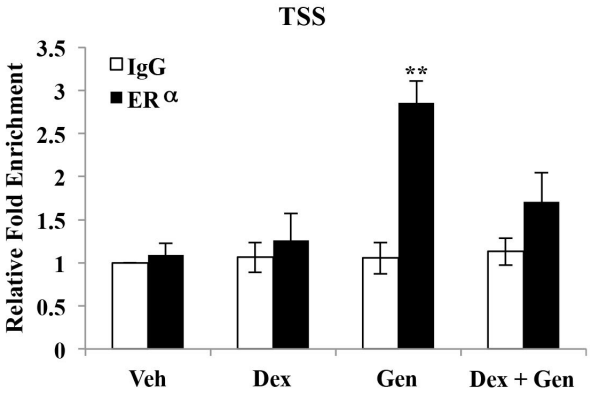
*Inclusion of select promoter elements

B









C